Factors Affecting Oxidative Phosphorylation by Subcellular Particles Isolated From Cotton Seedling Hypocotyls 1, 2
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Properly prepared mitochondrial fractions from plant sources are capable of incorporating inorganic phosphate into high energy bonds upon the oxidation of members of the tricarboxylic acid cycle (3, 6, 7). Oxidative phosphorylation has been demonstrated in preparations from a wide variety of plant sources.

This author reported that a high concentration of BSA3 in the grinding medium was necessary for cotton seedling particulate preparations to oxidize Krebs cycle intermediates (12). Because of this unusual requirement for BSA in the isolation technique, the study was extended to determine the influence of this material on oxidative phosphorylation. Certain other factors in the isolation and measurement technique were also considered.

Materials & Methods

Hypocotyl tissue from etiolated cotton seedlings (Gossypium hirsutum L. var. Acala 1517C) was obtained as reported previously (12). Tissue disruption was accomplished by either blending or mortar grinding. The medium to tissue ratios were 5 to 1 and 0.8 to 1 for disruption and washing, respectively, in blender preparations and 1.5 to 1 for both operations in the mortar procedure. The disruption medium consisted basically of 0.4 M sucrose and some concentration of BSA. Buffer, either phosphate or tris, was included for certain experiments. Washing and suspending media were the same as the disruption medium except when BSA was omitted from either or both of these steps. Other mechanics of preparation have been reported (12).

Oxygen consumption was measured at 30°C by the Warburg technique (13) and inorganic phosphate was determined by the method of Fisk and Subbarow (4). Reaction mixtures normally contained sucrose, phosphate at pH 7.0, MgSO4, ATP and/or AMP, glutamate, cytochrome c, glucose, hexokinase, and succinate. The flask center wells contained 0.2 ml 20% KOH, making a total initial volume of 3.0 ml. Air was the gas phase.

Hexokinase was initially placed in the side arm while the other reaction components were added to the main compartment. Immediately before placing the flasks on the manometers, 1.0 ml particulate preparation was added, hexokinase was tipped in, the contents were stirred, and 0.5 ml was removed from each flask and added to 2.0 ml cold 10% TCA. The TCA samples were centrifuged cold at 1,000 g for 15 minutes for initial phosphate aliquots. Flasks were equilibrated to bath temperature for 5 minutes and oxygen consumption was measured for the following 40-minute period. Another 0.5 ml sample was then immediately removed from each flask for final phosphate determination. The difference between initial and final phosphate concentrations was calculated as uptake of inorganic phosphate. Phosphate uptake was adjusted by a factor of 8/9 to account for the equilibration period before oxygen consumption was measured. Duplicate phosphate determinations were made on each 0.5-ml sample of flask contents. Each experiment included duplicate flasks, accompanied by a flask without substrate to correct for endogenous activity. Most experiments were run three times, but a few blender experiments were run two times.

Cytochrome c, ATP (99% +, Disodium, Chromatographed), AMP, BSA (Fraction V Powder), and hexokinase (28,000 K.M. units/g at 30°C) were obtained from Nutritional Biochemicals Corp.

Results

The data in table I were obtained from using various preparative media and two different methods of cell disruption. In blender preparations, the higher BSA concentration resulted in higher phosphorylation rates and P:O values when phosphate was included in the medium, but the reverse was true when phosphate was omitted. The results from equivalent BSA-containing media show reduced phosphate uptake from including phosphate at the high BSA level. In mortar preparations, more phosphate uptake was obtained with the higher BSA medium irrespective of phosphate presence. A decrease in

1 Manuscrt received for publication May 1, 1962.
2 Journal Series No. 177, Agricultural Experiment Station, New Mexico State University, University Park.
3 Abbreviations used in text: BSA, bovine serum albumin; tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid; AMP, adenylic acid; ATP, adenosine triphosphate.
phosphorylation was evident with phosphate-containing media except at the 2% BSA concentration, where it is likely that BSA itself was limiting. The apparent inhibitory effect of phosphate was particularly marked at 0.1 M. Tris buffer at this concentration did not show this character.

No differences were obtained between mortar and blender preparations with the 2% BSA-0.05 M phosphate medium. Both methods produced equally low P:O values. Mortar preparations were superior to blender preparations in phosphate uptake when an equivalent phosphate-high BSA medium was used. Where phosphate was omitted, blending resulted in increased phosphorylation at the lower BSA level, whereas mortar preparations were more active with the high BSA medium. Mortar grinding using a 0.4 M sucrose-5% BSA medium gave the highest phosphorylation rates of any of the combinations. This procedure was used routinely throughout the rest of the investigation. The data in table I show that there was no consistent pattern to indicate that extract pH was a particular factor in subsequent oxidation and phosphorylation rates.

Because of the BSA effect on activity of the preparations, experiments were carried out to determine the influence of washing, with and without BSA in the medium, and of including BSA in the reaction mixture. As shown in table II, washing with sucrose alone, rather than with sucrose-BSA, reduced phosphate uptake about half but had less effect on oxygen consumption. Unwashed preparations had extremely low phosphorylation rates but yet maintained rather high oxidation rates. The P:O values obtained show the desirability of washing and, in the particular case of cotton seedlings, the advantage of washing with a BSA medium. The results in table III compare routine preparations, where BSA was present throughout, with those where BSA was omitted from the suspending medium and then either added to or omitted from the reaction mixture. The data indicate that BSA was not a critical additive in these steps. The principal advantage of using BSA in cotton seedling preparations appears to lie in its protective action during grinding and washing.

<table>
<thead>
<tr>
<th>Method of preparation</th>
<th>Preparative medium**</th>
<th>pH of Extract</th>
<th>Oxygen uptake***</th>
<th>Phosphate uptake***</th>
<th>P:O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blender 0.05 M PO_4-2% BSA (w/v)</td>
<td>7.0</td>
<td>8.4</td>
<td>1.4</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>Blender 0.05 M PO_4-5% BSA</td>
<td>7.9</td>
<td>6.2</td>
<td>3.9</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td>Blender 1.5% BSA†</td>
<td>6.4</td>
<td>5.4</td>
<td>2.5</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>Blender 5% BSA</td>
<td>6.8</td>
<td>6.0</td>
<td>1.1</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>Mortar 0.05 M PO_4-2% BSA</td>
<td>6.9</td>
<td>6.2</td>
<td>1.3</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>Mortar 0.05 M PO_4-5% BSA</td>
<td>6.9</td>
<td>5.1</td>
<td>4.5</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>Mortar 0.1 M PO_4-5% BSA</td>
<td>7.0</td>
<td>6.5</td>
<td>3.0</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>Mortar 0.1 M tris-5% BSA</td>
<td>6.6</td>
<td>5.4</td>
<td>4.9</td>
<td>0.91</td>
<td></td>
</tr>
<tr>
<td>Mortar 2% BSA</td>
<td>6.0</td>
<td>6.0</td>
<td>0.0</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Mortar 5% BSA</td>
<td>6.4</td>
<td>5.0</td>
<td>5.6</td>
<td>1.12</td>
<td></td>
</tr>
</tbody>
</table>

* Reaction mixtures contained 0.143 M sucrose, 0.05 M glucose, 0.043 M glutamate, 0.05 M phosphate, 10^{-3} M MgSO_4, 5 \times 10^{-3} M AMP, 5 \times 10^{-3} M ATP, 3.25 \times 10^{-3} M cytochrome c, 0.02 M succinate, 0.7 mg/ml hexokinase, and either 7.1 or 17.8 mg/ml BSA, depending on preparative medium.

** Contained 0.4 M sucrose in addition to listed composition.

*** Microaoms oxygen and micromoles phosphate per flask in 40 minutes.

† Washed and suspended in sucrose-5% BSA medium.

<table>
<thead>
<tr>
<th>Washing medium</th>
<th>Oxygen uptake**</th>
<th>Phosphate uptake**</th>
<th>P:O</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4 M sucrose-5% BSA (w/v)</td>
<td>5.1</td>
<td>5.7</td>
<td>1.12</td>
</tr>
<tr>
<td>0.4 M sucrose</td>
<td>3.9</td>
<td>2.8</td>
<td>0.72</td>
</tr>
<tr>
<td>No wash</td>
<td>4.5</td>
<td>0.3</td>
<td>0.07</td>
</tr>
</tbody>
</table>

* Mortar preparations, ground and finally suspended in 0.4 M sucrose-5% BSA; reaction mixtures same as in table I with the higher BSA content.

** Microaoms oxygen and micromoles phosphate per flask in 40 minutes.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Oxygen uptake**</th>
<th>Phosphate uptake**</th>
<th>P:O</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA in suspending medium</td>
<td>5.0</td>
<td>3.0</td>
<td>0.60</td>
</tr>
<tr>
<td>No BSA in suspending medium</td>
<td>6.4</td>
<td>3.4</td>
<td>0.53</td>
</tr>
<tr>
<td>No BSA in suspending medium, no BSA in flasks</td>
<td>4.9</td>
<td>2.7</td>
<td>0.55</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Condition</th>
<th>Oxygen uptake**</th>
<th>Phosphate uptake**</th>
<th>P:O</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA in suspending medium</td>
<td>5.1</td>
<td>5.7</td>
<td>1.12</td>
</tr>
<tr>
<td>BSA added to flasks</td>
<td>6.4</td>
<td>3.4</td>
<td>0.53</td>
</tr>
<tr>
<td>No BSA in suspending medium, no BSA in flasks</td>
<td>4.9</td>
<td>2.7</td>
<td>0.55</td>
</tr>
</tbody>
</table>

* Mortar preparations, ground and washed in 0.4 M sucrose-5% BSA, suspended in 0.4 M sucrose, with or without 5% BSA (w/v); reaction mixtures same as table II except AMP only source of adenyate.

** Microaoms oxygen and micromoles phosphate per flask in 40 minutes.

Results obtained from varying phosphate concentration in the reaction mixture are presented in table IV. The concentration used routinely in this study, 0.05 M, was undoubtedly higher than that leading to
optimum sensitivity in phosphate determination and probably led to more variability than would be encountered at lower concentrations. This concentration, equivalent to that used by some investigators (5, 10, 14), resulted in higher oxidation and phosphorylation rates and P:O values than did the two lower concentrations. This agrees in principle with other reports, though phosphate concentrations were not directly comparable (8, 9, 11).

The data in table V show that adenylate presence and source had no appreciable effect on oxidation rates but did influence phosphorylation. Added ATP, as compared with AMP, resulted in reduced phosphate uptake. This differs from other reports of AMP being equivalent or superior to ATP in this respect (2, 9). Reduction of phosphorylation to a negligible quantity without added adenylate was to be expected (1, 2, 9). The omission of either or both hexokinase and glucose, components of the phosphate trapping system, did not influence oxidation (table VI). Phosphorylation was not appreciably reduced except when both were omitted, indicating that endogenous amounts of each were probably near marginal for optimum activity. The data in table VII show that 0.01 M and 0.001 M fluoride inhibited both oxidation and phosphorylation and did not appreciably raise the P:O values, an effect similar to that reported by Switzer and Smith (11). Improvement of apparent P:O values by fluoride by its action as an inhibitor of adenosine triphosphatase has been reported (2).

### Table IV

Effect of Phosphate Concentration in Reaction Mixture on Oxidative Phosphorylation*

<table>
<thead>
<tr>
<th>Phosphate conc</th>
<th>Oxygen uptake**</th>
<th>Phosphate uptake**</th>
<th>P : O</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 M</td>
<td>6.3</td>
<td>7.0</td>
<td>1.11</td>
</tr>
<tr>
<td>0.025 M</td>
<td>5.3</td>
<td>4.4</td>
<td>0.83</td>
</tr>
<tr>
<td>0.01 M</td>
<td>5.2</td>
<td>3.4</td>
<td>0.65</td>
</tr>
</tbody>
</table>

* Mortar preparations, ground, washed, and suspended in 0.4 M sucrose-5% BSA (w/v); reaction mixtures same as in table II except for varying phosphate concentrations.

** Microatoms oxygen and micromoles phosphate per flask in 40 minutes.

### Table V

Effect of Adenylate Source & Presence on Oxidative Phosphorylation*

<table>
<thead>
<tr>
<th>Adenylate source</th>
<th>Oxygen uptake**</th>
<th>Phosphate uptake**</th>
<th>P : O</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP + AMP</td>
<td>6.5</td>
<td>4.7</td>
<td>0.72</td>
</tr>
<tr>
<td>ATP</td>
<td>7.1</td>
<td>3.2</td>
<td>0.45</td>
</tr>
<tr>
<td>AMP</td>
<td>7.0</td>
<td>6.1</td>
<td>0.87</td>
</tr>
<tr>
<td>No adenylate</td>
<td>6.2</td>
<td>0.4</td>
<td>0.06</td>
</tr>
</tbody>
</table>

* Mortar preparations, ground, washed and suspended in 0.4 M sucrose-5% BSA (w/v); reaction mixtures same as in table II except for varying sources of adenylate.

** Microatoms oxygen and micromoles phosphate per flask in 40 minutes.

### Table VI

Effect of Added Hexokinase & Glucose on Oxidative Phosphorylation*

<table>
<thead>
<tr>
<th>Condition</th>
<th>Oxygen uptake**</th>
<th>Phosphate uptake**</th>
<th>P : O</th>
</tr>
</thead>
<tbody>
<tr>
<td>With hexokinase, with glucose</td>
<td>5.7</td>
<td>4.5</td>
<td>0.79</td>
</tr>
<tr>
<td>Without hexokinase, with glucose</td>
<td>5.9</td>
<td>3.7</td>
<td>0.63</td>
</tr>
<tr>
<td>With hexokinase, without glucose</td>
<td>5.6</td>
<td>4.3</td>
<td>0.77</td>
</tr>
<tr>
<td>Without both</td>
<td>5.3</td>
<td>1.0</td>
<td>0.19</td>
</tr>
</tbody>
</table>

* Mortar preparations, ground, washed, and suspended in 0.4 M sucrose-5% BSA (w/v); reaction mixtures same as in table II except for omissions of hexokinase and glucose, where indicated; sucrose added to compensate for glucose omitted.

** Microatoms oxygen and micromoles phosphate per flask in 40 minutes.

### Discussion

The generalized increase in phosphorylation rates and P:O values found with the higher level BSA medium is probably explained by the action of this material in offsetting the effect of a toxic material encountered in cotton seedlings. This protective influence with respect to oxidative activity of subcellular particles was reported earlier (26). This study indicates that the phosphorylation mechanism is probably more sensitive to the inhibiting substance than are the oxidative enzymes, since phosphate uptake was much more influenced by changes in BSA concentration. The protective action of BSA was very evident by its presence in the washing step but not in the reaction mixture. It is apparent that BSA or an equivalent material is a necessary ingredient in the preparative medium to obtain functional subcellular particles from cotton seedlings.

The present data indicate that mortar preparations are superior to blender preparations in subsequent phosphorylation capacity when no other factors seem to be limiting. Though not direct comparisons, similar activity has been reported for blender (1, 5) and mortar (2, 9, 10, 11, 14) preparations. Blending was...
done at line voltage here, however, rather than at reduced speeds, which may have led to damage to the phosphorylating system.

The influence of phosphate in the preparative medium on subsequent phosphate uptake is puzzling. Response to phosphate apparently varied with method of disruption and BSA concentration, indicating that some sort of an interaction existed which is not readily explainable.

Other factors which were considered produced results that were generally consistent with accepted mitochondrial behavior. The inability of added ATP to serve as an efficient source of adenylate as AMP should be examined further, since this is not in agreement with the work of Pierpoint (9) and Bonner and Millerd (2). The limited stimulation on phosphate uptake by added hexokinase observed here falls somewhere between the absence of an effect reported by some authors (1, 2) and a decided requirement reported by others (9, 11, 14).

P:O values found here ranged near unity under the most favorable conditions for isolation and measurement. Failure to achieve values closer to the theoretical 2 for succinate may be because of the omission of other needed cofactors and to the possibility of less than optimum conditions in the isolation procedure, due principally to the inhibitory factor found in cotton seedlings.

Summary

Subcellular particles capable of coupling inorganic phosphate uptake to the oxidation of succinate were isolated from etiolated cotton seedling hypocotyls by the inclusion of bovine serum albumin in the preparative medium. This material apparently protected the particles from inhibition by an unidentified toxic substance present in cotton seedlings. The protective action was evident only in the grinding and washing steps. Mortar preparations were superior to blender preparations in phosphate uptake under comparable conditions. Inorganic phosphate at 0.05 M in the flasks produced higher phosphorylation rates than did lower concentrations. Phosphorylation rates were higher with AMP rather than ATP as the added adenylate source. Hexokinase and glucose omitted singly had limited effects on phosphorylation but caused a sharp reduction when omitted simultaneously.

Summary

Fluoride inhibited both oxygen consumption and phosphate uptake and did not substantially alter apparent phosphorylation efficiency.

Literature Cited